CALCIUM HYPOCHLORITE AS A SEED STERILIZER*

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For certain physiological experiments seeds and plantlets free from active bacteria and fungi are necessary. Most attempts to secure such seeds or plantlets in any considerable number have resulted in failure, usually because of the harmful effects of the germicide, its low efficiency, or the complicated methods required for treatment of the seed. While it is recognized that no germicide will give perfect satisfaction under every condition, some are more effective in this respect than others.

The treatment of seed to remove bacteria and fungi, especially the latter, has been practiced for a considerable period of time; and while one investigator has secured fair results with a particular method, another one has considered it a failure, or nearly so, when tested on another kind of seed. As a result many methods have been proposed for seed sterilization with the final condition that most of them can not be relied upon to yield a very large percentage of sterile plantlets.

In the following compilation the methods of treatment employed by various investigators are summarized together with the seeds treated.

AUTHOR	SEED	DISINFECTANT
Brown and Escombe.	*Hordeum vulgaris.*	$CuSO_4$, 1%, one to two hours.
Combes.	Radish.	$HgCl_2$.
Czapek.	Corn.	$HgCl_2$, 1%, two minutes.
Godlewski and Polzeniusz.	*Pisum sativum, Vicia Faba, Triticum vulgare, Zea mays, Ricinus communis, Brassica napus.*	$HgCl_2$, 1/100%.
Grüss.	Grain.	HOH 48 hours. Then in $HgCl_2$ 45 minutes.
Hansteen.	*Zea mays* and others.	Washed in absolute alcohol then in $HgCl_2$ 1–1,000.
Kehler.	Peas, beans, blue lupine, yellow mustard, red clover, sugar beet, wheat, alfalfa, and others.	$HgCl_2$, CH_2O, various strengths of each.

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Laurent.	Corn, peas, lentils and other seeds.	$HgCl_2$ 1–500 alone and in combination with other substances.
Lefevre, J.	*Lepidium sativum, Ocimum minimum, Tropaeolum nanum.*	$HgCl_2$, 2 to 3 pts. per 1,000, 15 min. and longer.
Lewis and Nicholson.	Leguminous seed.	Phenol 5%, 50 min.
Lutz, L.	*Cucurbita maxima, Zea mays, Cucumis prophetarum, Helianthus annus* and others.	$HgCl_2$ 1–2,000, 5 min.
Maze and Perrier.	*Zea mays.*	$HgCl_2$ 1–1,000, 15 min.
Molliard, M.	*Raphanus sativus, Allium cepa, Ipomaea purpurea, Nasturtium officinale.*	Soak seed in sterile water until wrinkled then in abs. alc. for a short period, then 1 min. in 1% $HgCl_2$.
Nabokich, A.	*Zea mays* and others.	Bromine water 1–1,000 20 to 30 min.
Nobbe, F.	Wheat, timothy and others.	$CuSO_4$ various strengths for 24 hours.
AUTHOR	SEED	DISINFECTANT
Pinoy and Magrou.	*Sinapis alba, Lupinus albus, Zea mays, Orobus tuberosus.*	Br. water and H_2O_2.
Polowczow, W.	*Lepidium sativum, Lupinus luteus* and others.	Br. water.
Prazmowski, A.	Garden peas.	$HgCl_2$ 2%, then absolute alcohol and burned off alcohol.
Puriewitsch, K.	Endosperm of *Zea mays, Triticum sativum, Secale cereale, Hordeum distichum, Oryza sativa* and others.	CH_2O, 2½ parts—1,000, 3 min.
Robinson, T. R.	Alfalfa, crimson clover, garden pea, soy bean, wheat, oats, radish.	CH_2O, $HgCl_2$, H_2O_2 in various strengths.
Schroeder, H.	Wheat.	$AgNO_3$ 5%, 14 hrs.
Schroeder, H.	Wheat.	HNO_3, $AgNO_3$, $HgCl_2$, and other substances in combination.
Schulow, Iw.	Corn, peas, barley.	Br. water 20 min.
Staklasa, J.	*Hordeum distichon.*	$HgCl_2$ 0.1%.
Stoward, F.	*Hordeum, Zea* and *Ricinus.*	$CuSO_4$, $HgCl_2$, CH_2O, Sat. sol. chloroform. Sat. sol. toluene.
v. Ubisch, G.	Spore capsules of moss.	Alcoholic $HgCl_2$.
de Zeeuw, R.	*Lupinus albus, Pisum sativum, Triticum vulgare, Hor-*	H_2O_2, $HgCl_2$, cleaning fluid. Potassium dichromate, am-

deum vulgare, *Zea mays*, and *Sinapis alba*.	monium persulfat, and bromine water.

It will be seen from the above compilation, to which others might be added, that about two dozen methods have been used. Of these mercuric chloride, alcohol, formalin, hydrogen peroxide, or combinations of these have served in the main as the germicide.

The writer tried various of the above methods and finding them unsatisfactory resorted to the use of other substances among which was bleaching powder (calcium hypochlorite). The favorable results which have been secured with bleaching powder and the many requests for the method have prompted the author to present this brief description. The method is simple: Ten grams of commercial chloride of lime (titrating 28 percent chlorine) is mixed with 140 cc. of water. The mixture is then allowed to settle for five or ten minutes and the supernatant liquid decanted off or filtered. The solution or filtrate which contains about 2 percent chlorine is used as the disinfectant. Dilutions from this known strength may be used as well as the full strength. The volume of solution employed should be about five times or more the volume of the seed.

In order to find the exposure at which the ability for germination is affected seeds were placed in sterile test tubes and covered with a one percent chlorine solution, obtained as above, for different lengths of time. Germination tests were then made with the seed which were removed at these different times and compared with untreated seed germinated in the same way.

Seeds were also removed from the disinfecting solution at various intervals and tests made with respect to freedom from bacteria or fungus organisms. The most trustworthy tests were made by planting the seed on the surface of peptone agar and into bouillon. Tubes thus prepared were kept at room temperature, at 30° C., and 37° C. Observations were made on these tubes at intervals up to four weeks. In addition to these tests of sterility many plants which were germinated on agar were used in experimental work where sterility was required and was apparently maintained for several months.

In making transfers the seed was removed from the disinfecting solution by means of a small hand-wrought spoon, momentarily drained and sown in the culture vessel. No attempt was made to remove completely the disinfectant from the seed since it does not seem to interfere with the germination unless the period of treatment

is exceedingly long or a noticeable amount is carried over with the seed. In making the transfers, however, an effort was made to leave behind as much of the germicide as possible. That the quantity of disinfecting material carried over with the seed into the bouillon was not sufficient to act as a germicide was proven by placing along with some of the treated seed in the bouillon an untreated seed. In every case within three days there was ample bacterial growth.

With large seeds such as corn, beans, peas, and wheat, only those seeds were used which appeared normal and seemed capable of producing vigorous plants. With other seed, such as timothy, alfalfa, clover, etc., no such selection was made. The number of seeds placed in each series of six to ten tubes varied from one to three with corn and from four to one hundred with other seeds. The number, however, varied with the size of the seed and the amount of available agar surface in the test tube. In several cases as many as 4,000 timothy seeds were treated at a time and transferred to a single container.

The following table presents some of the data.

Seed	Time Required for Sterilizing Seed. Hours	Time Required to Cause Injury to Seed. Hours
Zea Mays L.	8	18
Medicago sativa L.	6	18
Triticum vulgare Vill.	15	20–22
Phleum pratense L.[2]	8	above 22
Brassica Rapa L.[3]	4	" 11
Pastinaca sativa L.[3]	9	" 11
Petroselinum sativum Hoffm.[3]	2 : 15	" 11
Viola tricolor L.[3]	1	" 11
Cosmos bipinnatus Cav.[3]	5	" 10
Brassica oleracea L.[3]	2	" 11
Trifolium pratense L.[3]	24	" 24
Linum usitatissimum L.[3]	10	" 14
Fagopyrum esculentum Moench.[3]	No success at all	" 40
Avena sativa L.[2]	7 : 30	" 15
Solanum tuberosum L.[4]	No success at all	

[2] Seeds hulled before treatment.

[3] Tested only on peptone agar and at room temperature.

[4] Cuttings of tuber.

The data show that the time required in most cases for sterilizing seed and the exposure necessary to produce injury are many hours apart. This allows considerable latitude with respect to the time during which the disinfectant may be allowed to act. It is evident

also that the different seeds are not sterilized in the same length of time, alfalfa requiring about six hours while wheat requires more than twice as long.

Other tests of the efficiency of the method have been made. In one experiment thirty or more carefully selected seeds of each of the different kinds used were placed in test tubes and covered with a calcium hypochlorite solution containing approximately two percent chlorine. After treatment the seeds were transferred into test tubes on the surface of a medium which contained tap water and one percent each of agar and saccharose. After germination of the seed each tube was inoculated with a pure culture of *Bacillus radicicola* and placed in the greenhouse where it remained 45 days before examination. The seeds used and the time of treatment of each are as follows:

Seed	Time of Treatment
Medicago sativa L.	7 hours
" *hispida* Gaertn.	7
" *media* ?	7
" *falcata* L.	7
" *lupulina* L.	7
Melilotus alba Desr.	7
" *indicata* (L.) All.	7
" *officinalis* (L.) Lam.	7
Vicia villosa Roth.	6½
" *sativa* L.	6½
" *Faba* L.	3
Lathyrus sylvestris L.	5
" *sativus* L.	4

The above treated seeds were planted in 184 test tubes which were reopened when inoculated. On examination only three tubes showed signs of contamination. Two of these were contaminated with molds while the other was with a bacterial organism.

In addition to the foregoing tests the following data on the efficiency of the method were contributed by Dr. Knudson from experiments made on the organic nutrition of plants. These data are valuable because they indicate the thorough sterilization of the seed as is shown by the freedom of cultures from contaminations even after 30 days or more of culture. The duration of immersion of the seed in the disinfectant and the culture media employed are indicated in the following table.

Seed	Immersion Hours	Media. Pfeffer's Solution ½ Strength to which was Added in Separate Lots
Phleum pratense L.	7½	Saccharose, glucose, levulose, maltose and lactose each 2%.
Zea Mays L.	10	Glucose, levulose, maltose and saccharose 2%. One series in the laboratory and one in the greenhouse.
Vicia villosa Roth. (100 seeds)	6	Lactose, maltose, saccharose, 1% each. Sterilization not always successful.
Raphanus sativus L.	6	Lactose, maltose, dextrose, and saccharose, each 1%.
Brassica oleracea L.	11	One series in greenhouse and one in laboratory on saccharose, maltose, levulose, lactose, each in four concentrations from one tenth percent to 2%.
Linum usitatissimum L.	11	Maltose and lactose each 1%.
Pisum sativum L.	4½	Saccharose, lactose, maltose, glucose each 1% and checks.

Satisfactory sterilization was secured in every case with the exception of the vetch seed. In certain experiments where only a few vetch seeds were employed no contaminations occurred in the cultures except occasionally where it was clearly evident that contamination was due to causes other than the failure of the hypochlorite. In certain experiments on the influence of sugars on respiration, 100 seeds were required for each culture chamber. Failure to sterilize occurred in 20 out of 40 trials and in each case it was due to introduction of one or more dead seed along with others.

The considerable number of experiments made in which this method of seed sterilization has been employed and in which sterile plantlets have been secured and maintained over a period of thirty days or more conclusively demonstrate the efficiency of the method as an aid for securing sterile plantlets. The ease of operation and the fact that the solution does not injure the seed except after long exposure to the hypochlorite solution make the method particularly desirable. The effect of the solution is probably due to the hypochlorous acid, as suggested by Hooker, which acts as the toxic agent. In conclusion it should be added that the method not only offers a way for securing sterile plantlets for physiological experiment but also for eradicating such plant diseases as may be controlled by treating the seed.

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